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TITLE

ADENOVIRUS E4ORF4 PROTEIN FOR INDUCING CELL DEATH

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TITLE OF THE INVENTION *(280 characters max)*

ADENOVIRUS E4orf4 PROTEIN FOR INDUCING CELL DEATH

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PROVISIONAL APPLICATION FILING ONLY

ADENOVIRUS E4orf4 PROTEIN FOR INDUCING CELL DEATH

BACKGROUND OF THE INVENTION

(a) Field of the Invention

The invention relates to a pharmaceutical agent(s) to induce cell death in any diseases which involve inappropriate cell survival.

(b) Description of Prior Art

Replication of human adenoviruses in terminally differentiated epithelial cells requires an efficient mechanism to induce cellular DNA synthesis to permit replication of viral DNA and production of progeny virus. Human adenoviruses infect and kill epithelial cells very efficiently. Cell death occurs by apoptosis and virus spread occurs through endocytosis by surrounding cells. Products of early region 1A (E1A) induce cell DNA synthesis and are largely responsible for cell transformation by adenoviruses. E1A produces two major mRNAs of 13S and 12S which encode proteins of 289 and 243 residues (289R and 243R, respectively) that are identical except for a central 46-amino acid sequence, termed conserved region 3 or CR3. Two additional regions present in the common sequence encoded by exon 1 of both E1A mRNAs are also conserved in all human serotypes and have been termed CR1 and CR2 (see Fig. 1A). E1A products induce DNA synthesis through complex formation between CR2 and CR1 and the retinoblastoma tumor suppressor pRB and related p107 and p130 proteins, or between the amino terminus and CR1 and the transcriptional modulator p300 and possibly related proteins (Corbeil, H.B. et al., 1994, *J. Virol.*, **68**:6697-6709). E1A-289R also activates expression of the early viral transcription units E2, E3 and E4, and certain cellular genes at least in part through interactions with transcription factors and basal transcription machinery requiring CR3 (Teodoro, J.G., et al.,

1995, *Oncogene*, **11**:467-474). In addition to CR3, transactivation of the E4 promoter has also been shown to rely to some degree on two regions encoded by the second exon of 13S mRNA, termed auxiliary regions 1 and 2, or AR1 and AR2. Production of stably transformed cells requires early region 1B (E1B) which encodes polypeptides of 19 and 55kDa that are individually capable of cooperating with E1A via separate but additive pathways (McLorie, W. et al., 1991, *J. Gen Virol.*, **72**:1467-1471).

Considerable evidence indicates that a major function of E1B proteins in lytic infection and cell transformation is to suppress cytotoxic effects and apoptosis induced by expression of E1A. Without E1B, the toxicity of E1A products results in the death of E1A-transformed cells and a reduction in the yield of progeny due to the early demise of productively infected cells. E1A proteins can cause apoptosis by a process mediated by the tumor suppressor p53 (Teodoro, J.G., et al., 1995, *Oncogene*, **11**:467-474), which controls growth arrest and programmed cell death pathways (Teodoro, J.G., et al., 1995, *Oncogene*, **11**:467-474). Expression of E1A products results in the elevation of p53 levels. The 55kDa E1B protein binds to p53 and blocks both p53-mediated activation of gene expression and apoptosis (Teodoro, J.G., et al., 1994, *J. Virol.*, **68**:776-786). The 19kDa E1B protein appears to suppress apoptosis by a mechanism that is functionally analogous to that of the cellular proto-oncogene product Bcl-2 (Nguyen, M. et al., 1994, *J. Biol. Chem.*, **269**:16521-16524). Cells infected with adenovirus mutants which fail to express the 19kDa protein display enhanced cytotoxicity and extensive degradation of both cellular and viral DNA into nucleosome sized fragments (McLorie, W. et al., 1991, *J. Gen Virol.*, **72**:1467-1471; Teodoro,

J.G., et al., 1995, *Oncogene*, **11**:467-474). At later times, even in the presence of E1B proteins, infected cells suffer apoptotic death and viral progeny spread to neighboring cells through endocytosis of cell fragments. In addition to the induction of DNA synthesis and cell transformation, the large 289-residue (289R) E1A protein also transactivates expression of all early viral genes, including early regions 1A, 1B, 2, 3 and 4 (reviewed in Teodoro, J.G., et al., 1995, *Oncogene*, **11**:467-474).

Recently our group showed that in the absence of E1B, E1A products also induce p53-independent apoptosis (Teodoro, J.G., et al., 1995, *Oncogene*, **11**:467-474). Our results indicated that such apoptotic cell death was only induced by the 289R E1A protein. Furthermore, when p53-null mouse cells constitutively expressing E1A products were infected by an adenovirus vector lacking the entire E1A and E1B coding regions but containing early regions E2, E3 and E4, rapid cell death due to apoptosis was observed (Teodoro, J.G., et al., 1995, *Oncogene*, **11**:467-474). We have shown that 289R induces apoptosis in p53-null mouse and human cells, and that such p53-independent cell death requires the expression of another early viral gene (Teodoro, J.G., et al., 1995, *Oncogene*, **11**:467-474). Genetic analysis indicated that neither E2 nor E3 products were necessary and that one or more E4 proteins are responsible (Marcellus, S. et al., 1996, *J. Virol.*, **70**:6207-6215 (1996)). E4 encodes several mostly unrelated proteins whose functions are only partially understood. These results indicated that the role of E1A-289R may be to transactivate expression of an additional early transcript whose product actually induces p53-independent apoptosis. In the present

studies we report that one or more E4 gene products appear to be responsible for such cell killing.

It would be highly desirable to be provided with a pharmaceutical agent for induction of apoptosis when such induction is useful in the treatment of human diseases which involve inappropriate cell survival.

SUMMARY OF THE INVENTION

In accordance with the present invention, a genetic approach to identify the role of individual E4 proteins in the induction of p53-independent apoptosis was used. Our results indicated that the E4orf4 protein is one of the proteins responsible. Thus E4orf4 is a powerful inducer of p53-independent cell death.

One aim of the present invention is to develop E4orf4 as a pharmaceutical agent for induction of apoptosis when such induction is useful in the treatment of human diseases which involve inappropriate cell survival.

In accordance with the present invention there is provided a pharmaceutical agent for induction of apoptosis for the treatment of human diseases which involve inappropriate cell survival, which comprises E4orf4, an analog or a biologically active fragment thereof.

In accordance with the present invention there is also provided a pharmaceutical composition for the treatment of human diseases which involve inappropriate cell survival, which comprises a therapeutic amount of E4orf4, an analog or a biologically active fragment thereof in association with a pharmaceutical carrier.

In accordance with the present invention, the expression "diseases which involve inappropriate cell survival" include, without limitation, diseases caused by HIV, herpes and/or other viral infections, Alzheimer's, cancer, arthritis, lupus among others.

The pharmaceutical agent of the present invention allows for the selective killing of cells that are prevented from dying by a virus or as a consequence of a disease state. Thus, the pharmaceutical agent of the present invention only kills the inappropriately surviving cells, such as cancer cells or viral infected cells. This results in a substantially side effect free therapy for the patient.

The pharmaceutical agent of the present invention includes, without limitation, any adenovirus of any serotype E4 death protein (E4-ADP) products, fragment thereof and mimetic peptides of this protein products.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1A shows the encoding amino acid sequences of the Ad5 E1A mutants;

Fig. 1B summarizes relevant adenovirus mutants;

Fig. 2 shows the pattern of DNA fragmentation by Ad5 mutants in the absence of p53;

Figs. 3A and 3B are graphs of the viability of infected normal and Bcl-2 expressing SAOS-2 cells;

Fig. 4 is the gel analysis of the induction of DNA fragmentation by E1A mutants;

Fig. 5 is a graph of the analysis of viability of SAOS-2 cells infected with E1A mutants;

Fig. 6 is a gel analysis of the induction of DNA degradation in the absence of E3;

Fig. 7 is a graph of the role of E4 products in p53-independent cell killing;

Fig. 8 is a gel analysis of the analysis of DNA degradation in the absence of E4; and

Figs. 9A and B are graphs of the analysis of p53-independent cell killing in the absence of E4 products;

Fig 10 shows the encoding amino acid sequences of the orf proteins;

Fig. 11 is a graph of the role of E4 products in p53-independent cell killing;

Fig. 12 is a graph of the role of E4orf4 in p53-independent cell killing; and

Fig. 13 is a graph showing that E4orf4 when expressed by transient transfection of an E4orf4-encoding plasmid induces cytotoxicity in p53-null cells, as judged by the low expression of a co-transfected reporter plasmid encoding luciferase, relative to a non-cytotoxic inducing control plasmid encoding E4orf1.

DETAILED DESCRIPTION OF THE INVENTION

In the absence of E1B, the 289- and 243-residue (289R and 243R) E1A products of human adenovirus type 5 (Ad5) induce p53-dependent apoptosis. However, our group has shown recently that the 289R E1A protein is also able to induce apoptosis by a p53-independent mechanism (Teodoro et al., 1995, *Oncogene*, 11:467-474). Preliminary results suggested that p53-independent cell death required expression of one or more additional adenovirus early gene products. Here we show that both the E1B-19kDa protein and cellular Bcl-2 inhibit or significantly delay p53-independent apoptosis. Neither early regions E2 or E4 appeared to be necessary for such cell death. Analysis of a series of E1A mutants indicated that mutations in the transactivation domain and other regions of E1A correlated with E1A-mediated transactivation of E4 gene expression. Furthermore, p53-deficient human SAOS-2 cells infected with a mutant which expresses E1B but none of the E4 gene products remained viable for considerably longer times than those infected with wt Ad5. In addition, an adenovirus vector lacking both E1 and E4 was unable to induce DNA

degradation and cell killing in E1A-expressing cell lines. These data showed that an E4 product is essential for E1A-induced p53-independent apoptosis.

Cells and viruses

Human Saos-2 cells (ATCC HTB 85) and 10(1) mouse embryo fibroblast-derived cells which are both deficient for p53 expression were cultured on 60mm-diameter dishes (Corning Glass Works, Corning, N.Y.) in Dulbecco's modified MEM (D-MEM) supplemented with 10% fetal calf serum (FCS) as were both NIH-3T3 and CHO cells. The cell line Saos-2/Bcl-2(3g4) which stably expresses Bcl-2 was derived for this study from Saos-2 cells by selection with G418 as was the control line Saos-2/Neo(2a2). A1.A3, A1.A6 and A1.A12 mouse embryo fibroblast cell lines expressing Ad5 E1A proteins, and Hy.A3 hygromycin-selected control lines, have been described previously (Lowe, S.W. et al., 1994, *Proc. Natl. Acad. Sci. U.S.A.*, **91**:2026-2030), and were cultured in D-MEM containing 10% FCS and 100 µg/mL of hygromycin. Normally cells were infected with mutant or wild-type (wt) Ad5 at a multiplicity of 100 pfu per cell. Ad5 E1A mutants are illustrated in Fig. 1A and include deletion mutants dl1101 (residues 4-25 deleted), dl1143 (38-60), dl1107 (111-123), dl1108 (124-127), dl1143/08 (38-60 plus 124-127) and dl1132 (224-238) which have all been described previously (Marcellus, S. et al., 1996, *J. Virol.*, in press). Proteins encoded by some of the mutants used in the present studies have been presented, including the residues removed in deletion mutants. CR1, CR2, CR3, AR1 and AR2 have also been indicated. A new E1A mutant was constructed as termed $AR1^{-}/E1B^{-}$, which lacks the entire AR1 region (residues 189-200) and also fails to express E1B products. Mutant $AR2^{-}/E1B^{-}$ was generated by introducing dl1132, which lacks residues 224-238,

into a background that fails to express E1B proteins. Mutant AR1$^-$/AR2$^-$/E1B$^-$ represents a combination of the latter two mutants. Additional E1A mutants containing single amino acid substitutions at various sites within CR3 were produced by subcloning appropriate restriction enzyme fragments from mutant E1A cDNA plasmids into genomic viral DNA, followed by rescue into virus to form mutants AD147VL (Val-147 converted to Leu), AD177CS, and AD185SG. All other mutants have been summarized in Fig. 1B. A list providing the names and defects of E1B and other mutants has been presented. Two were produced previously by our group (McLorie, W. et al., 1991, *J. Gen Virol.*, **72**:1467-1471) and fail to express E1B proteins of 19kDa (originally termed pm1716/2072 but now called E1B/19K) and 55kDa (originally pm2019/2250, now E1B/55K$^-$). Mutant 12S/E1B$^-$ (originally dl520E1B$^-$) produces only the E1A-243R protein encoded by the 12S mRNA and no E1B products. Mutant E1B$^-$ which expresses both major E1A products but neither the 19kDa or 55kDa E1B species was described previously (Teodoro, J.G., et al., 1995, *Oncogene*, **11**:467-474), and a similar mutant that expresses only 289R in the absence of E1B, termed 13S/E1B$^-$, was prepared for the present studies. A series of E1A mutants (dl1101/E1B$^-$, dl1107/E1B$^-$, AD147VL/E1B$^-$, etc.) which express no E1B products was also produced by introducing E1A mutations into mutant E1B$^-$ which expresses both 289R and 243R E1A products but no E1B (Teodoro, J.G., et al., 1995, *Oncogene*, **11**:467-474). The presence of mutations in all mutants was confirmed by DNA sequencing, restriction enzyme digestion, or Southern blotting. Ad5 vectors used in this study included AdLacZ in which the E1 (E1A + E1B) region was replaced with the E. coli gene lacZ under the CMV promoter, and Ad5dl70-8 which was generated by

cotransfection of plasmids pAB7 and pBHG10 and which lacks both E1 and the entire E3 region. Adenovirus vector AdRSVβgal.11 which lacks the entire E1 and E4 regions was a gift of Douglas Brough. In addition, some experiments were carried out with human adenovirus type 2 (Ad2) mutant dl1019 which contains deletions that eliminate expression of all E4 products and which was propagated on W162 monkey cells, as described previously (Bridge, E. et al., 1989, *J. Virol.*, **63**:631-638). Other E4 mutants (Bridge, E. et al., 1989, *J. Virol.*, **63**:631-638) have been summarized in Fig. 10.

DNA fragmentation

Low molecular weight DNA was isolated from mock- or Ad5-infected cells as described in Teodoro et al. (1995, *Oncogene*, **11**:467-474). For such experiments, 60mm-diameter plates of cells were harvested at 40 h post-infection and lysed in pronase lysis buffer comprised of 10mM Tris-HCl (pH 8) containing 5mM EDTA, 100mM NaCl, and 1 mg/mL (w/v) pronase to which SDS was added to 0.5% (w/v). Cell lysates were incubated at 37°C for 2 h and NaCl was added to a final concentration of 1M. Samples were then incubated overnight at 4°C and centrifuged at 15,000 x g for 30 min. Extracted nucleic acids were treated with RNAase A and analyzed on 1% agarose gels stained with ethidium bromide.

Cell viability assays

Cells were infected with wt or mutant virus in 24-well plates containing cells at about 80% confluence. At various times following infection adherent and non-adherent cells were pooled and viability was assessed by Trypan Blue™ exclusion. At least 300 cells were counted at each time point.

Measurement of E1A-mediated transactivation of the adenovirus E4 promoter

Transactivation assays were performed using NIH 3T3 or CHO cells plated at a density of 2 x 10^5 cells on 60 mm-diameter dishes. The E4 reporter plasmid was E4-CAT containing the E4 promoter upstream of the chloramphenicol acetyltransferase (CAT) gene. Transient co-transfections were performed by the calcium phosphate precipitation method using 2.5 µg of reporter plasmid DNA and 2.5 µg of DNA from plasmids expressing wt or mutant E1A products as described in Marcellus et al., 1996. Cells were glycerol shocked after 12h and then harvested 36 h later. CAT assays were performed using cell extracts containing equal amounts of β-galactosidase activity. The amount of activity was quantified from TLC plates using a Fujix Bas 2000™ Phosphorimager.

E1A-induced p53-independent apoptosis is inhibited by both the E1B-19kDa protein and cellular Bcl-2

Previous studies indicated that whereas both major Ad5 E1A products could induce apoptosis in cells expressing p53, only the 289R E1A protein could do so in cells lacking p53 (Teodoro, J.G., et al., 1995, *Oncogene*, 11:467-474). Fig. 2 shows the pattern of DNA fragmentation in $p53^-$ mouse 10(1) cells infected by various Ad5 mutants. 10(1) cells which fail to express p53 were infected with various Ad5 mutants, or they were mock-infected, and at 40 h p.i., low molecular weight DNA was analyzed by agarose gel electrophoresis. The contents of individual lanes are as indicated in Fig. 2. Extracts from mock-infected cells (lane 1) and those infected with wt Ad5 (lane 2) which expresses E1B products displayed reduced levels of extracted low molecular weight DNA and little or no degraded DNA, as did those from cells infected with mutant $E1B/55K^-$ (lane 4) which produces the E1B-19kDa protein but not

the E1B-55kDa product. With cells infected with mutant E1B$^-$ which synthesizes both the 289R and 243R E1A proteins but which produces no E1B products (lane 6), large amounts of DNA were extracted and high levels of nucleosome-sized DNA fragments were evident. Similar results were also obtained with cells infected with E1B/19K$^-$ (lane 5) which produces the E1B-55kDa species but not the 19kDa protein. Induction of DNA degradation in these p53$^-$ cells did not occur following infection with 12S/E1B$^-$ (lane 3) which produces only E1A-243R and no E1B, but it did occur with 13S/E1B$^-$ (lane 7) which yields only E1A-289R in the absence of E1B products. Thus as shown previously, E1A-289R but not 243R induces p53-independent apoptosis in the absence of E1B proteins. In addition the results indicated that the E1B 19kDa polypeptide but not the 55kDa E1B product is able to protect against apoptosis induced by E1A in the absence of p53.

To examine the specificity of inhibition of apoptosis further, studies were conducted to determine if the cellular Bcl-2 protein is also able to prevent p53-independent apoptosis as several previous studies had shown that Bcl-2 and the E1B-19kDa protein may be functionally similar (Nguyen, M. et al., 1994, *J. Biol. Chem.*, **269**:16521-16524). Human Saos-2 cells which are defective for synthesis of p53 were transfected with cDNAs encoding the human Bcl-2 protein and the neomycin resistance marker and several cell lines were selected using G418. One such Bcl-2 expressing clone, termed Saos-2/Bcl-2(3g4), and a control SAOS-2 clone, SAOS-2/neo(2a2) selected only for resistance to G418, were infected with wt Ad5, mutants 12S/E1B$^-$ or E1B/19K$^-$, or were mock-infected, and cell viability assays were conducted at various times after infection. In Fig. 3, p53-deficient human Saos-2/neo(2a2) cells (panel A) or

Saos-2/Bcl-2(3g4) which express human Bcl-2 constitutively (panel B) were mock-infected or infected with wt, E1B/19K$^-$ or 12S/E1B$^-$ and were tested for viability by a Trypan Blue™ exclusion assay at various times following infection, as described above. Results have been presented as the logarithm of the % viable cells, and symbols are as indicated in Fig. 3. Fig. 3A shows that SAOS-2/neo(2a2) control cells were killed by the E1B/19K$^-$ virus that expresses E1A-289R, but those infected with wt or 12S/ E1B$^-$ remained almost as viable as mock-infected cells during the test period. Fig. 3B shows that with Saos-2/Bcl-2(3g4) cells which stably express high levels of Bcl-2, little cell death was induced by the E1B/19K$^-$ virus. Similar results were obtained with three other control and Bcl-2 producing SAOS-2 cell lines. Thus like the E1B-19kDa protein, Bcl-2 also blocks E1A-induced p53-independent apoptosis.

Role of E1A domains in p53-independent apoptosis.

To investigate the regions of E1A products involved in causing p53-independent cell death, p53$^-$ mouse 10(1) cells were infected with Ad5 mutants which fail to express E1B and which harbor a variety of defects at various regions of the E1A molecule. Extracts were harvested and analyzed on gels to determine the extent of degradation of low molecular weight DNA. In Fig. 4, an experiment in p53-deficient 10(1) cells similar to that described in Fig. 2 was performed using a series of Ad5 E1A mutants defective in expression of E1B products. The contents of individual lanes are as indicated in Fig. 4. Fig. 4 shows that again mutant E1B/19K$^-$ (lane 3) induced DNA degradation whereas such did not occur with wt Ad5 (lane 2) or mock-infected cells (lane 1). Mutants which affected the E1A transactivation function associated with CR3

all failed to induce DNA degradation. These included 12S/E1B$^-$ (lane 8), and point mutants AD147VL/E1B$^-$, AD171CS/E1B$^-$ and AD185SG/E1B$^-$ (lanes 9 to 11, respectively) which carry single residue substitutions at critical residues in CR3 that eliminate E1A transactivation activity. In addition, deletion of AR1 or both AR1 and AR2 (AR1$^-$ /E1B$^-$ and AR1$^-$ /AR2$^-$ /E1B$^-$ in lanes 12 and 14) also eliminated DNA degradation whereas removal of AR2 alone (AR2$^-$ /E1B$^-$ in lane 13) had little effect. Interestingly, mutants in CR2 which eliminate complex formation with pRB and related proteins (dl1107/E1B$^-$ and dl1108/E1B$^-$ in lanes 5 and 6) had no effect on the induction of DNA degradation, whereas those that eliminated binding of p300 by removal of the N-terminus (dl1101/E1B$^-$ in lane 4) or a portion of CR1 as well as the pRB binding site (dl1143/08/E1B$^-$ in lane 7) no longer caused this effect. These results suggested that E1A-induced p53-independent apoptosis required the CR3 transactivation domain, AR1, and the regions necessary for binding of p300 but not pRB-related proteins. Fig. 5 shows that similar results were obtained with these mutants in cell killing assays. An experiment similar to that described in Fig. 3 was carried out in SAOS-2 cells infected with various E1A mutants defective in expression of E1B products. Results have been presented as the logarithm of the % viable cells, and symbols are as indicated in Fig. 5. Cell death was induced by the E1B/19K$^-$ virus which expresses both E1A products and by dl1107/E1B$^-$. Mutant AR2$^-$/E1B$^-$ which lacks AR2 also killed, but was consistently less toxic that the former viruses. All other mutants affecting CR3, AR1 and the p300 binding sites failed to kill significantly during the test period.

Activation of E4 expression and apoptosis.

The requirement for AR1 suggested that E4 products might somehow be involved in the induction of p53-independent apoptosis as this region is not important in the activation of other early viral transcription units. Studies were therefore carried out to examine the pattern of E1A transactivation of the E4 promoter in which plasmid DNA encoding various mutants forms of E1A-289R was co-transfected into NIH-3T3 or CHO cells along with DNA from E4-CAT, a construct that encodes CAT under the control of the Ad5 E4 promoter. Table 1 shows that in addition to CR3, activation of the E4 promoter required AR1 and to some extent AR2.

Table 1
E4 Transactivation by E1A Mutants

E1A Mutant	Mutation	Region Affected	E4 CAT * Activity (% wt ±S.D.)
wt	none	none	100
dl1101	Δ4-25	N-terminus	30 ± 11
dl1104	Δ48-60	CR1	40 ± 5
dl1107	Δ111-123	CR2	85 ± 5
dl1108	Δ124-127	CR2	81 ± 14
dl520	Δ140-185	CR3	10 ± 7
$AR1^-$	Δ189-200	AR1	25 ± 7
$AR2^-$(pm1132)	Δ224-238	AR2	64 ± 16

* CHO or 3T3 cells were transfected with plasmid DNA encoding various E1A mutants and CAT under the Ad5 E4 promoter.

Cell extracts were assayed for CAT activity as described above. The activity has been expressed as a % of that obtained with wt. Three independent assays were done for each mutant.

In addition, regions at the N-terminus and in CR1 involved in binding of p300 also were of some im-

portance. These results closely paralleled the pattern of E1A-induced p53-independent apoptosis and suggested that E4 products might be involved.

E2 and E3 products are not required for apoptosis

It was unlikely that E2 products were responsible for the induction of p53-independent apoptosis for two reasons. First, in addition to CR3, complex formation involving CR2 and the pRB family of proteins activates E2 expression, and CR2 was shown to be of little importance in cell killing. Second, reasonably high levels of expression of E2 proteins are known to be induced by the E1A-243R protein which is completely unable to induce p53-independent apoptosis. Thus experiments were carried out to determine if any E3 products were involved. The A1.A3 mouse embryo fibroblast cell line lacking p53 but expressing Ad5 E1A proteins, and Hy.A3 hygromycin-selected $p53^-$ control cells (Lowe, S.W. et al., 1994, *Proc. Natl. Acad. Sci. U.S.A.*, **91**:2026-2030), were infected with wt Ad5, the $E1B/19K^-$ virus, adenovirus vector AdlacZ which contains lacZ in place of E1A and E1B, or with vector Ad5d170-8 which lacks both the entire E1 and E3 regions. Cell extracts were assayed for the presence of degraded DNA as before. Fig. 6 shows that high levels of DNA degradation were induced in A1.A3 cells with the $19K^-$ mutants as well as both adenovirus vectors. Cell lines expressing 289R and 243R E1A proteins constitutively, or the Hy.A3 non-expressing control cell line, were mock-infected or infected with wt Ad5 or adenovirus vectors AdLacZ or Ad5d170-8. After 40 h, DNA was extracted and analyzed by agarose gel electrophoresis. The contents of individual lanes are as indicated in Fig. 6. Similar results were also obtained with two other similar E1A-expressing cell lines, A1.A6 and A1.A12. Fig. 6 also shows that in the control cells

lacking constitutive E1A expression, only the E1B/19K$^-$ virus induced DNA degradation. These results indicated that E3 products were not required for induction of p53-independent apoptosis by E1A under these conditions.

E4 proteins are essential for p53-independent apoptosis

To determine directly if E4 products are involved in the induction of cell death, as suggested by experiments described above, two approaches were taken. In the first, human p53$^-$ SAOS-2 cells were infected with wt Ad5 or Ad2, or with Ad2 mutant dl1019 which produces no E4 proteins (Bridge, E. et al., 1989, *J. Virol.*, **63**:631-638), or they were mock-infected. Although such viruses express E1B proteins and thus are protected from E1A-induced apoptosis, it was thought that if E4 products were essential for p53-independent cell death, some difference in long term cell survival might be observed, and thus at various times up to 10 days, infected cultures were tested for cell viability. Fig. 7 shows that cells infected either by wt Ad5 or Ad2 virus began to die at about 100h p.i., and by 240h p.i. almost all of the cells were dead. SAOS-2 cells were mock-infected or infected with wt Ad5 or Ad2, or dl1019 which expresses no E4 products. At various times up to 10 days cell viability was assessed by Trypan Blue™ exclusion. Data have been expressed as % cell viability and symbols are as indicated in the Fig. 7. Such was not the case with dl1019-infected cells which remained almost as viable as mock-infected cells even 10 days after infection. These results indicated that an E4 product was involved in cell killing in the absence of p53. This idea was confirmed in experiments involving infection of E1A-expressing p53$^-$ A1.A3 cells with the adenovirus vector AdRSVβgal.11 in which both the E1 and E4 regions had been completely

deleted. Fig. 8 shows that in control Hy.A3 $p53^-$ cells which do not express E1A, only the E1B/19K$^-$ Ad5 mutart caused DNA degradation, and neither wt , 12S/E1B$^-$, nor the AdRSVβgal.11 vector had any significant effect. Cell lines expressing 289R and 243R E1A proteins constitutively, or the Hy.A3 non-expresser control cell line, were mock-infected or infected with wt Ad5, E1B/19K$^-$, 12S/E1B$^-$, or the adenovirus vector AdRSVβgal.11 which lacks both E1 and E4. After 40 h, DNA was extracted and analyzed by agarose gel electrophoresis. The contents of individual lanes are as indicated in the Fig. 8. With A1.A3 cells, both the E1B/19K$^-$ and 12S/E1B$^-$ induced DNA degradation, but the AdRSVβgal.11 vector still had little effect. Similar results were obtained with the other two sister cell lines, A1.A6 and A1.A12 discussed above. The ability of this virus to induce apoptosis in A1.A3 cells was analyzed further in cell killing experiments. Cell lines expressing 289R and 243R E1A proteins constitutively (panel A), or the Hy.A3 non-expresser control cell line (panel B), were mock-infected or infected with wt Ad5, E1B/19K$^-$, 12S/E1B$^-$, or the adenovirus vector AdRSVβgal.11. At various times after cell viability was assessed by Trypan Blue™ exclusion. Data have been expressed as % cell viability and symbols are as indicated in the Fig. 9. Fig. 9A shows that in the Hy.A3 control cells, only the E1B/19K$^-$ virus induced cell death, whereas in A1.A3 cells both the E1B/19K$^-$ and 12S/E1B$^-$ viruses did so. However in both cases the AdRSVβgal.11-infected cells remained as fully viable as mock-infected cultures. These data thus confirmed that an E4 product is responsible for E1A-induced p53-independent cell death.

RESULTS AND DISCUSSION

To identify which E4 product is responsible for induction of E1A-dependent p53-independent apoptosis, p53-null mouse 10(1) cells were infected with wt Ad5 or with mutants carrying deletions in various portions of the E4 region (see Fig. 10). Fig. 11 shows that as shown above, cells in cultures infected by the wt virus began to die by about 125 hours following infection, and death was almost complete by 240 hours. Little cell death was observed in mock-infected cultures or in those infected by mutant dl1019 which lacks the entire E4 region. Cell death similar to that found with wt was observed with E4 mutants dl1013 which expresses E4orf6 and E4orf4, whereas little death occurred with mutant dl1010 which expresses all E4 products except E4orf6. These results indicated that cell death occurred only when the E4orf6 protein was expressed and did not take place during the course of the experiment in its absence. Thus it is clear that expression of the E4orf6 protein may be important for the p53-independent apoptosis induced by the 289R E1A product.

Further experiments using mutants dl1014 which expresses E4orf4 only, dl1015 which expresses E4orf4 and E4orf3, dl1011 which expresses no E4 products, as well as much infected cells, are illustrated in Fig. 12. These results indicate that expression of E4orf4 alone induced cell death. Thus, E4orf4 is capable of inducing p53-independent cell death.

This conclusion was confirmed in a second type of experiment in which E4orf4 was expressed alone in the absence of alter viral proteins using transient transfection with E4orf4 plasmid DNA in combination with a reporter plasmid encoding luciferase (Fig. 13). In this experiment E4orf4 alone was shown to have

potent cytotoxic activity as judged by the substantial reduction in luciferase activity.

It has been known for some time that adenovirus E1A products induce DNA degradation, rapid cell death and other hallmarks of apoptosis when expressed in the absence of E1B products whose major role in lytic infection and transformation is to suppress E1A toxicity. Both the 289R and 243R E1A proteins are able to induce apoptosis through p53-dependent pathways.

E1A proteins also induce apoptosis in cells lacking p53 (Teodoro, J.G., et al., 1995, *Oncogene*, **11**:467-474). We found that this p53-independent apoptosis was elicited only by the 289R E1A protein, and preliminary evidence suggested that expression of one or more additional early viral genes regulated by E1A-289R was required. The present experiments indicated that the E1B-55kDa protein is unable to block this effect, but both the E1B-19kDa product and the cellular suppressor of apoptosis Bcl-2 significantly inhibited this response.

The major goal of this work was to identify which early viral transcription units were required to induce cell death in the absence of p53. Results obtained with E1A mutants clearly indicated the CR3 is important. Furthermore, CR3-mediated transactivation activity appeared to be required, as several point mutants in CR3 which were known to eliminate transactivation of target genes were defective for induction of DNA degradation and cell killing. Of great interest were results obtained with mutants with defects outside CR3. Mutant dl1108 which lacks the core binding site for pRB and related proteins induced p53-independent apoptosis like wt. However, mutant dl1101 which binds pRB at reasonably normal levels but fails to bind the p300 transcriptional modulator was totally defective. These

results may suggest that interactions between p300 and 289R are essential to institute cell death pathways. Another possibility was offered by results obtained with two additional mutants with defects in the AR1 and AR2 regions encoded by the second exon of the 13S E1A mRNA. The AR1-defective mutant was unable to induce p53-independent apoptosis, and that lacking AR2 was somewhat impaired. These results corresponded exactly to the relative abilities of these mutant E1A molecules to transactivate the E4 promoter. We also found that dl1101 was partially defective for transactivation of E4, thus suggesting both that E4 products might be involved in induction of cell death and that interactions of 289R with p300 may reflect more a requirement for transactivation of E4 transcription than a direct role in apoptosis. This question will only be answered by further experiments using a new series of mutants.

Early regions E2, E3 and E4 encode a variety of products which could play some role in cell death. E2 proteins are largely involved in viral DNA synthesis. However, it is unlikely that any play an essential role in cell death. First, E2 transcription requires not only CR3, but also the formation of complexes with pRB which result in the activation of the E2F family of transcription factors and E2 gene expression. Our results clearly indicated that complex formation with pRB was not essential for apoptosis. Second, the adenovirus vector AdRSVβgal.11 contains a wt E2 region and yet was defective for induction of p53-dependent apoptosis in E1A-expressing cells. The E3 region encodes several proteins which affect virus-host interactions, however, the adenovirus vector Ad5dl70-8 was fully capable of inducing apoptosis in E1A expressing p53-deficient cells. As discussed above, the pattern of apoptosis observed with E1A mutants suggested that the

early viral proteins asscciated with cell death are encoded by E4. Direct evidence that an E4 protein is responsible was obtained from experiments in which the pattern of death was observed in p53-null SAOS-2 cells infected by wt Ad5 or a mutant defective in E4 expression. Because E1B products were expressed by these viruses, cell death occurred only at late times, but the observation that E4 mutant-infected cells displayed considerably retarded death clearly implicated an E4 product in the death process. Final confirmation came from results with the AdRSVβgal.11 adenovirus vector which was defective for cell killing. This virus was unable to induce DNA degradation or cell killing in p53-deficient cells expressing E1A.

It is likely that the E4orf4, or the adenovirus E4orf4 Death Protein (E4orf4-ADP), is capable of killing human cells following productive infection by adenoviruses. Cell death could be induced early after infection following expression of E1A proteins, however, p53-dependent apoptosis, which is induced directly by E1A, is blocked by expression of both the 55kDa and 19kDa E1B proteins. Following expression of the E4orf4-ADP, infected cells could die by p53-independent apoptosis were it not for the E1B-19kDa product which blocks cell death until late in infection (Marcellus, S. et al., 1996, *J. Virol.*, **70**:6207-6215 (1996)). Cell death may eventually occur because the levels of the E4orf4-ADP become too elevated for suppression by E1B-19K.

The E4orf4-ADP could be of use in killing cells that accumulate in several disease states, including some auto-immune disorders and cancer. Such cells fail to die by apoptosis and, at least in many cancers, one reason is because many cancer cells lack or express a mutant form of p53. These cells would, however, be

susceptible to killing by the E4orf4-ADP. Further studies will be necessary to define the specificity of cell killing and the mechanism of induction of apoptosis by the E4orf4-ADP.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

I CLAIM:

1. A pharmaceutical agent for induction of apoptosis for the treatment of human diseases which involve inappropriate cell survival, which comprises E4orf4, an analog or a biologically active fragment thereof.

2. A pharmaceutical composition for the treatment of human diseases which involve inappropriate cell survival, which comprises a therapeutic amount of E4orf4, an analog or a biologically active fragment thereof in association with a pharmaceutical carrier.

3. A pharmaceutical composition for the treatment of human diseases which involve inappropriate cell survival, which comprises a therapeutic amount of a compound which induces protein phosphatase 2a in association with a pharmaceutical carrier.

4. The composition of claim 3, wherein said compound is an agonist of E4orf4 or a compound which mimic E4orf4 activity.

5. A pharmaceutical composition for the treatment of human diseases which involve inappropriate cell survival, which comprises a therapeutic amount of a compound which induces apoptosis or other cytotoxic effects analogous to E4orf4 biological activity in association with a pharmaceutical carrier.

ABSTRACT OF THE INVENTION

The present invention relates to a pharmaceutical agent for induction of apoptosis for the treatment of human diseases which involve inappropriate cell survival, which comprises E4orf4, an analog or a biologically active fragment thereof. There is also provided a pharmaceutical composition for the treatment of human diseases which involve inappropriate cell survival, which comprises a therapeutic amount of E4orf4, an analog or a biologically active fragment thereof in association with a pharmaceutical carrier.

Applicant or Patentee: Philip E. BRANTON et al.
Serial or Patent No.: _____ Atty. Dkt. No.: 1770-161"US" FC/
Filed or Issued: _____
For: ADENOVIRUS E4orf4 PROTEIN FOR INDUCING CELL DEATH

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS
[(37 CFR 1.9(f) and 1.27 (b)] - INDEPENDENT INVENTOR

As a below named inventor, I hereby declare that I qualify as an independent inventor as defined in 37 CFR 1.9(c) for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States Code, to the Patent and Trademark Office with regard to the invention entitled ADENOVIRUS E4orf4 PROTEIN FOR INDUCING CELL DEATH described in

(X) the specification filed herewith
( ) application serial no. _____, filed _____
( ) patent no. _____, issued _____

I have not assigned, granted, conveyed or licensed and am under no obligation under contract or law to assign, grant, convey or license, any rights in the invention to any person who could not be classified as an independent inventor under 37 CFR 1.9(c) if that person had made the invention, or to any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

Each person, concern or organization to which I have assigned, granted, conveyed, or licensed or am under an obligation under contract or law to assign, grant, convey, or license any rights in the invention is listed below:

( ) no such person, concern, or organization
(X) persons, concerns or organizations listed below*

*NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. [37 CFR 1.27]

FULL NAME McGill University
ADDRESS 845 Sherbrooke Street West, Montreal, Quebec, Canada H3A 1B1
( ) INDIVIDUAL ( ) SMALL BUSINESS CONCERN (X) NONPROFIT ORGANIZATION

FULL NAME _____
ADDRESS _____
( ) INDIVIDUAL ( ) SMALL BUSINESS CONCERN ( ) NONPROFIT ORGANIZATION

FULL NAME _____
ADDRESS _____
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I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. [37 CFR 1.28(b)]

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF INVENTOR	SIGNATURE	DATE
Philip E. Branton		15/Oct/1996
Richard C. Marcellus		15/oct/1996
Jose G. Teodoro		15 Oct 1996
Gordon C. Shore		15 Oct 1996
Josée N. Lavoie		15 oct/1996

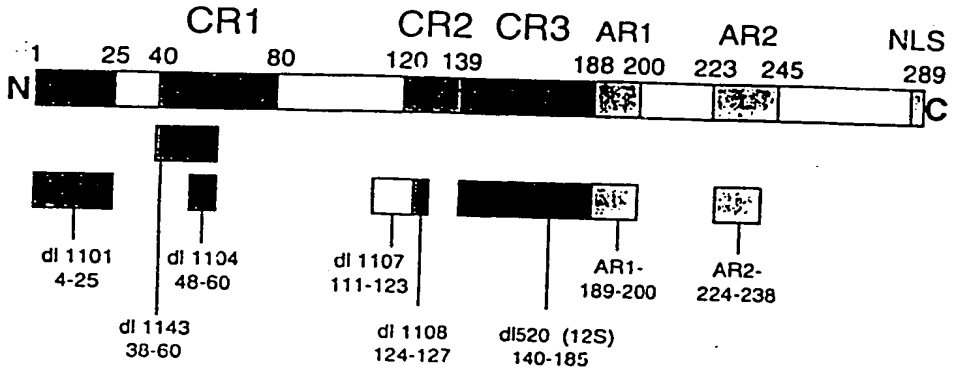

CR1
CR2 CR3
AR1
AR2
NLS
1
25 40
80
120 139
188 200
223 245
289
N
C
dl 1101
4-25
dl 1143
38-60
dl 1104
48-60
dl 1107
111-123
dl 1108
124-127
dl520 (12S)
140-185
AR1-
189-200
AR2-
224-238

Fig. 1A

Virus Mutant	Description
wt Ad5	wt E1A (12S &13S mRNAs), wt E1B. In some cases *dl* 309 which has a partial deletion of E3 was used
12S / E1B-	12S E1A mRNA only, no E1B expression
13S / E1B-	13S E1A mRNA only, no E1B expression
E1B / 55K-	wt E1A (12S &13S mRNAs), no E1B 55K expression, wt E1B 19K
E1B / 19K-	wt E1A (12S &13S mRNAs), no E1B 19K expression, wt E1B 55K
dl 1101 / E1B-	12S/13S E1A mRNAs, E1A mutation as in fig. 1A, no E1B expression
dl 1104 / E1B-	12S/13S E1A mRNAs, E1A mutation as in Fig. 1A, no E1B expression
dl 1107 / E1B-	12S/13S E1A mRNAs, E1A mutation as in Fig. 1A, no E1B expression
dl 1108 / E1B-	12S/13S E1A mRNAs, E1A mutation as in Fig. 1A, no E1B expression
dl 1143 / 08 / E1B-	12S/13S E1A mRNAs, E1A mutation as in Fig. 1A, no E1B expression
AR1- / E1B-	12S/13S E1A mRNAs, E1A mutation as in Fig. 1A, no E1B expression
AR2- / E1B-	12S/13S E1A mRNAs, E1A mutation as in Fig. 1A, no E1B expression
AR1- / AR2- / E1B-	12S/13S E1A mRNAs, E1A mutation as in Fig. 1A, no E1B expression
AD147VL / E1B-	13S E1A only, E1A point mutation in CR3, no E1B expression
AD171CS / E1B-	13S E1A only, E1A point mutation in CR3, no E1B expression
AD185SG / E1B-	13S E1A only, E1A point mutation in CR3, no E1B expression
dl 1019	wt E1A, E1B, E2 and E3, no E4 expression, in Ad2
AdLacZ	no E1A or E1B expression, wt E2, E3 and E4
Ad5*dl*70-8	no E1A, E1B or E3 expression, wt E2 and E4
AdRSVβ-gal.11	no E1A, E1B or E4 expression, wt E2 and E3

Fig. 1B

Fig. 2

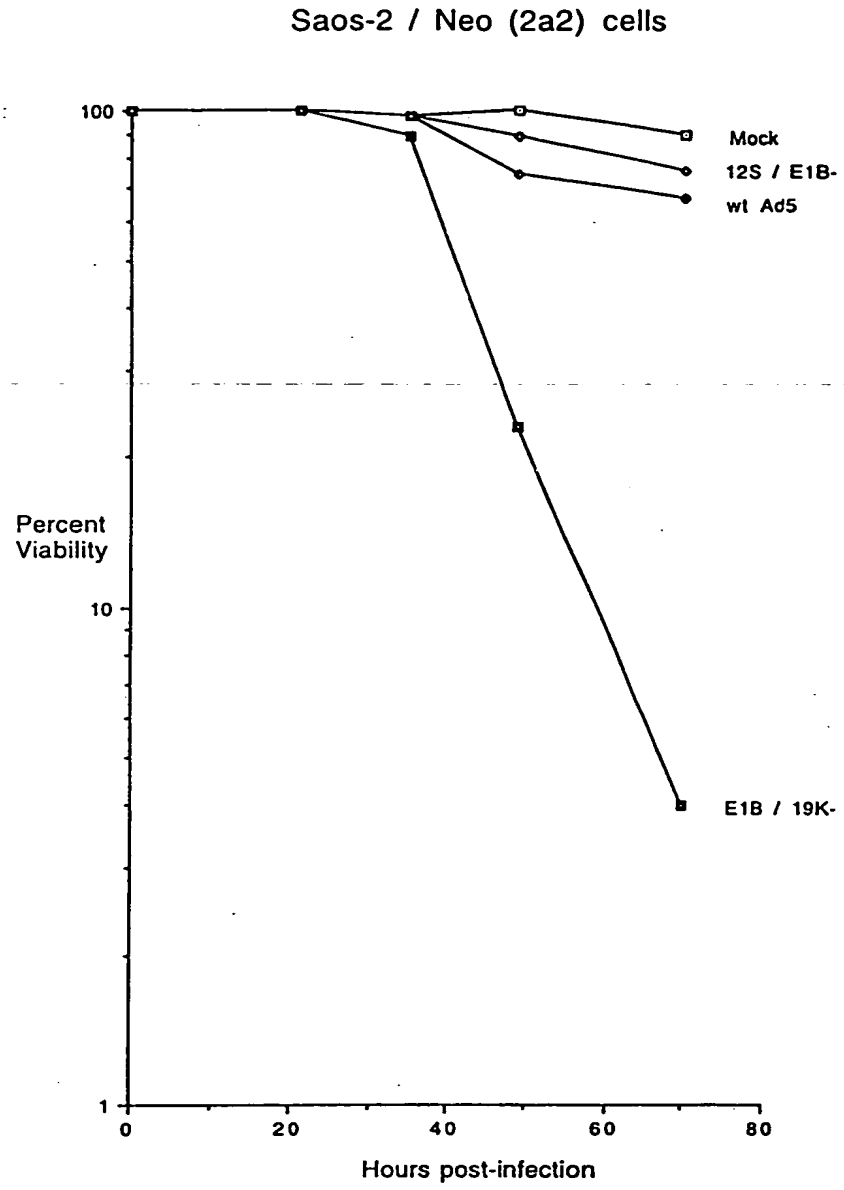

Saos-2 / Neo (2a2) cells
100
10
1
Percent Viability
0
20
40
60
80
Hours post-infection
Mock
12S / E1B-
wt Ad5
E1B / 19K-

Fig. 3A

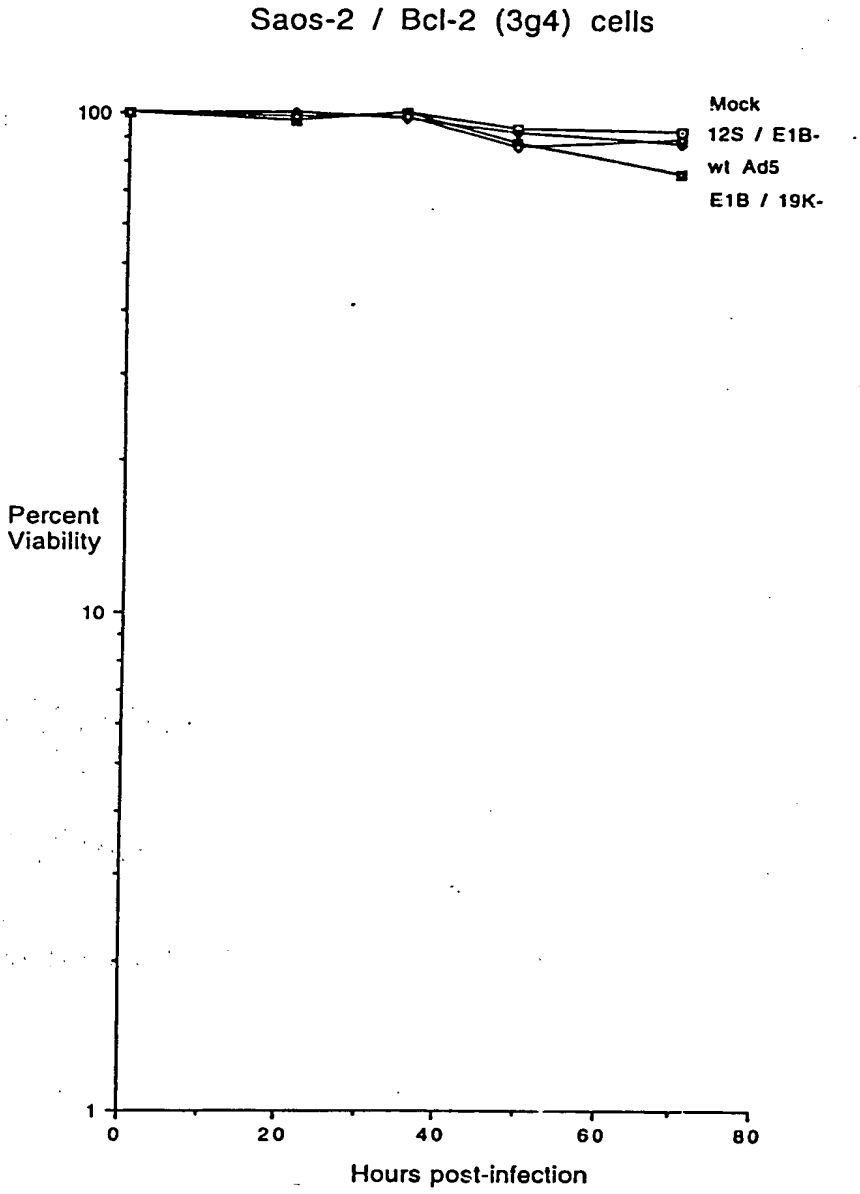

Saos-2 / Bcl-2 (3g4) cells
Mock
12S / E1B-
wt Ad5
E1B / 19K-
Percent Viability
100
10
1
0
20
40
60
80
Hours post-infection

Fig. 3B

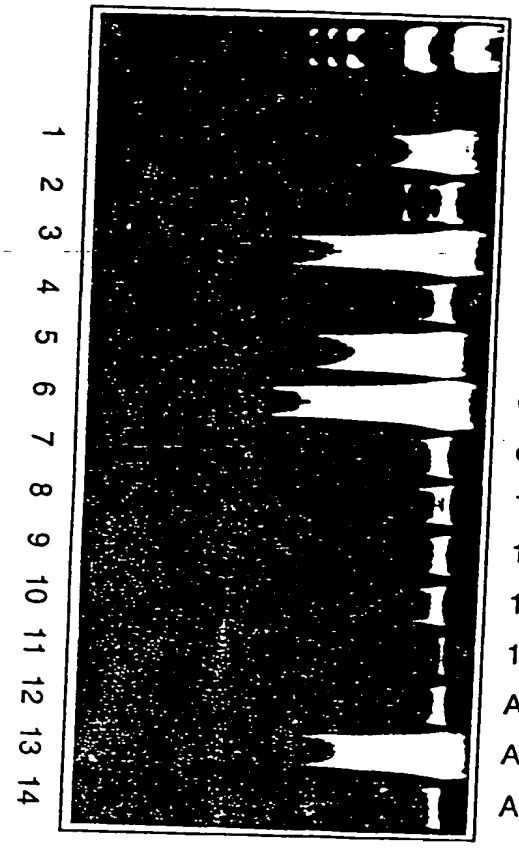

1 2 3 4 5 6 7 8 9 10 11 12 13 14
Markers
Mock
wt Ad5
E1B / 19k-
dl 1101 / E1B-
dl 1107 / E1B-
dl 1108 / E1B-
dl 1143 / 08 / E1B-
12S / E1B-
147VL / E1B-
171CS / E1B-
185SG / E1B-
AR1- / E1B-
AR2- / E1B-
AR1- / AR2- / E1B-

Fig. 4

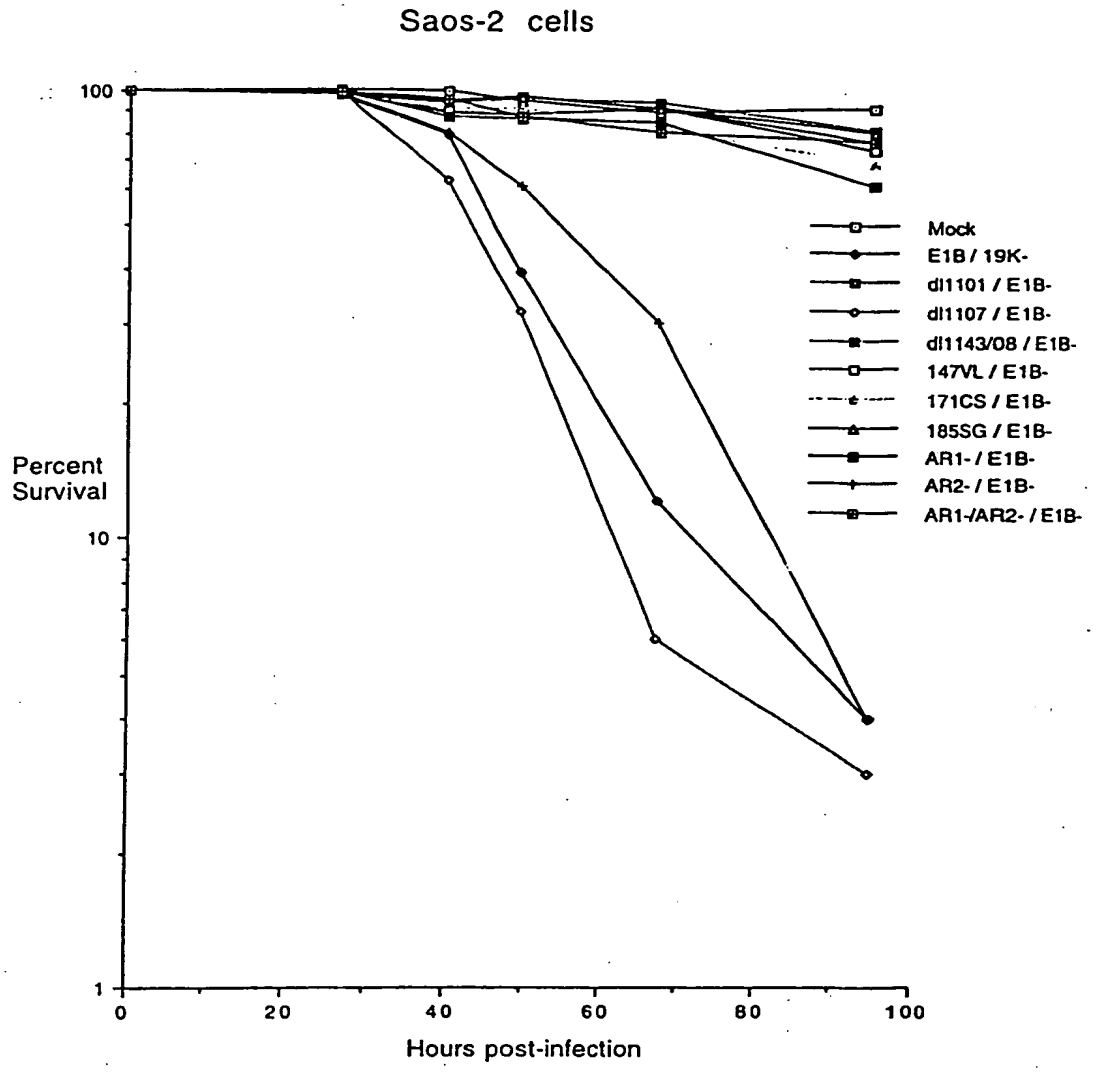

Saos-2 cells
100
10
1
Percent Survival
0
20
40
60
80
100
Hours post-infection
Mock
E1B / 19K-
dl1101 / E1B-
dl1107 / E1B-
dl1143/08 / E1B-
147VL / E1B-
171CS / E1B-
185SG / E1B-
AR1- / E1B-
AR2- / E1B-
AR1-/AR2- / E1B-

Fig. 5

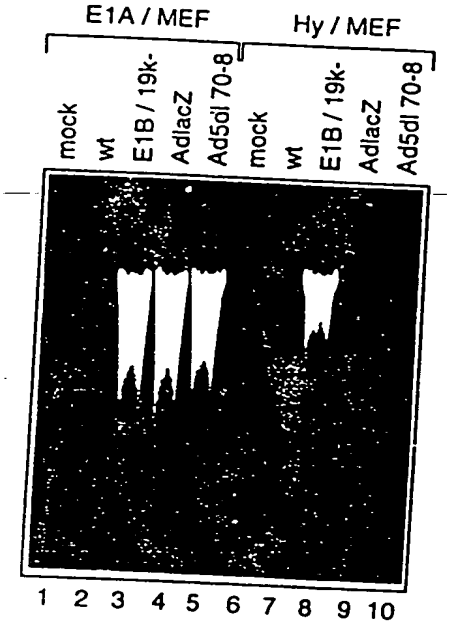

E1A / MEF
Hy / MEF
mock
wt
E1B / 19k-
AdlacZ
Ad5dl 70-8
mock
wt
E1B / 19k-
AdlacZ
Ad5dl 70-8
1 2 3 4 5 6 7 8 9 10

Fig. 6

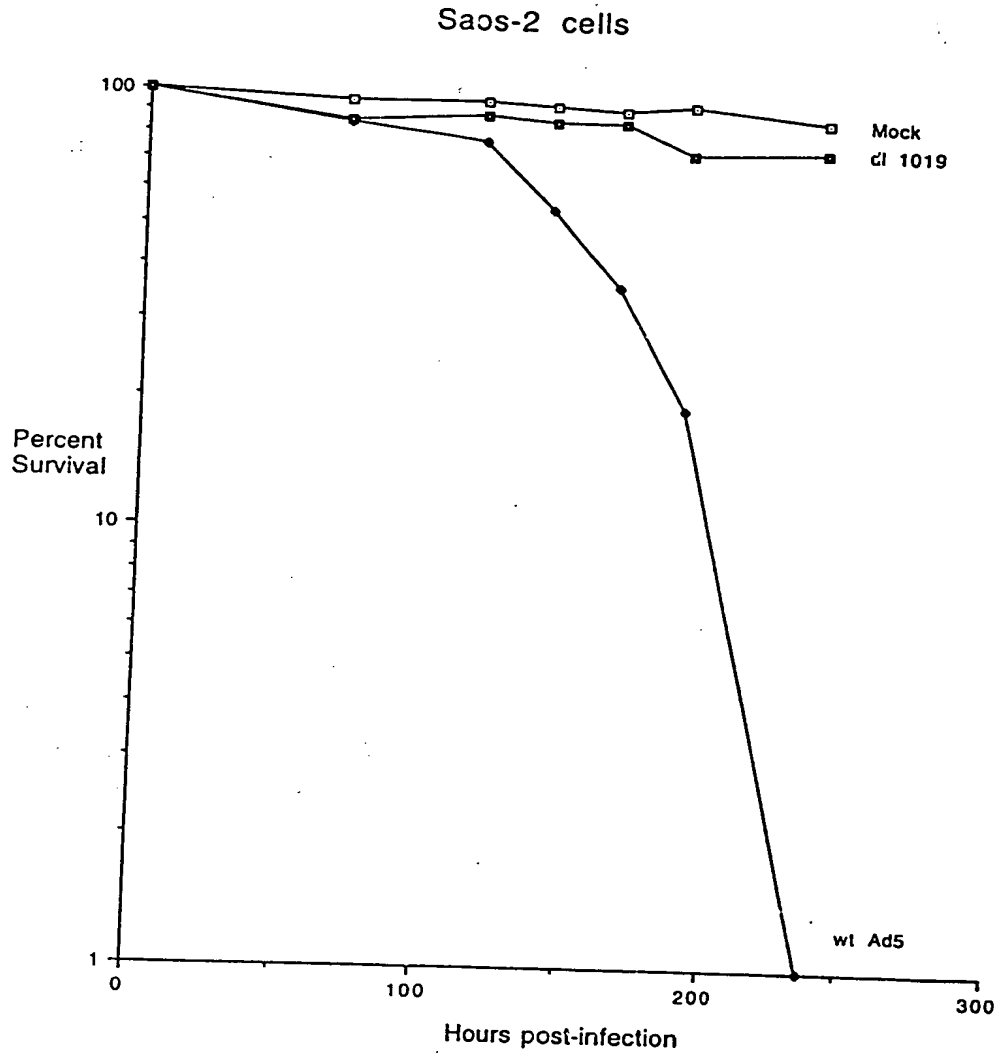

Saos-2 cells
100
10
1
Percent Survival
Mock
dl 1019
wt Ad5
0
100
200
300
Hours post-infection

Fig. 7

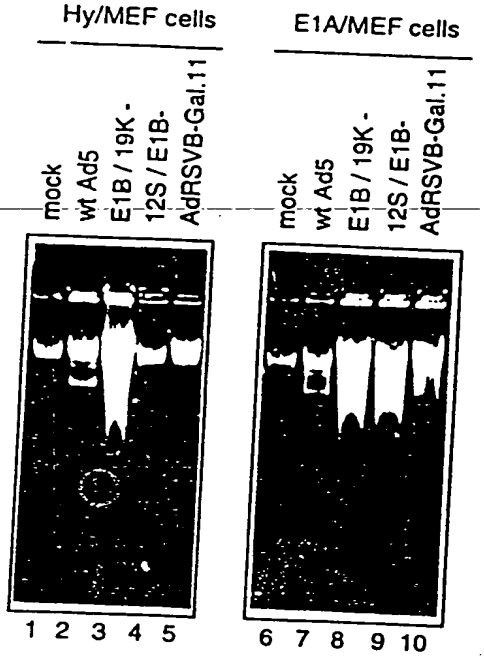

Hy/MEF cells
E1A/MEF cells
mock
wt Ad5
E1B / 19K - 12S / E1B-AdRSVB-Gal.11
mock
wt Ad5
E1B / 19K - 12S / E1B-AdRSVB-Gal.11
1 2 3 4 5
6 7 8 9 10

Fig. 8

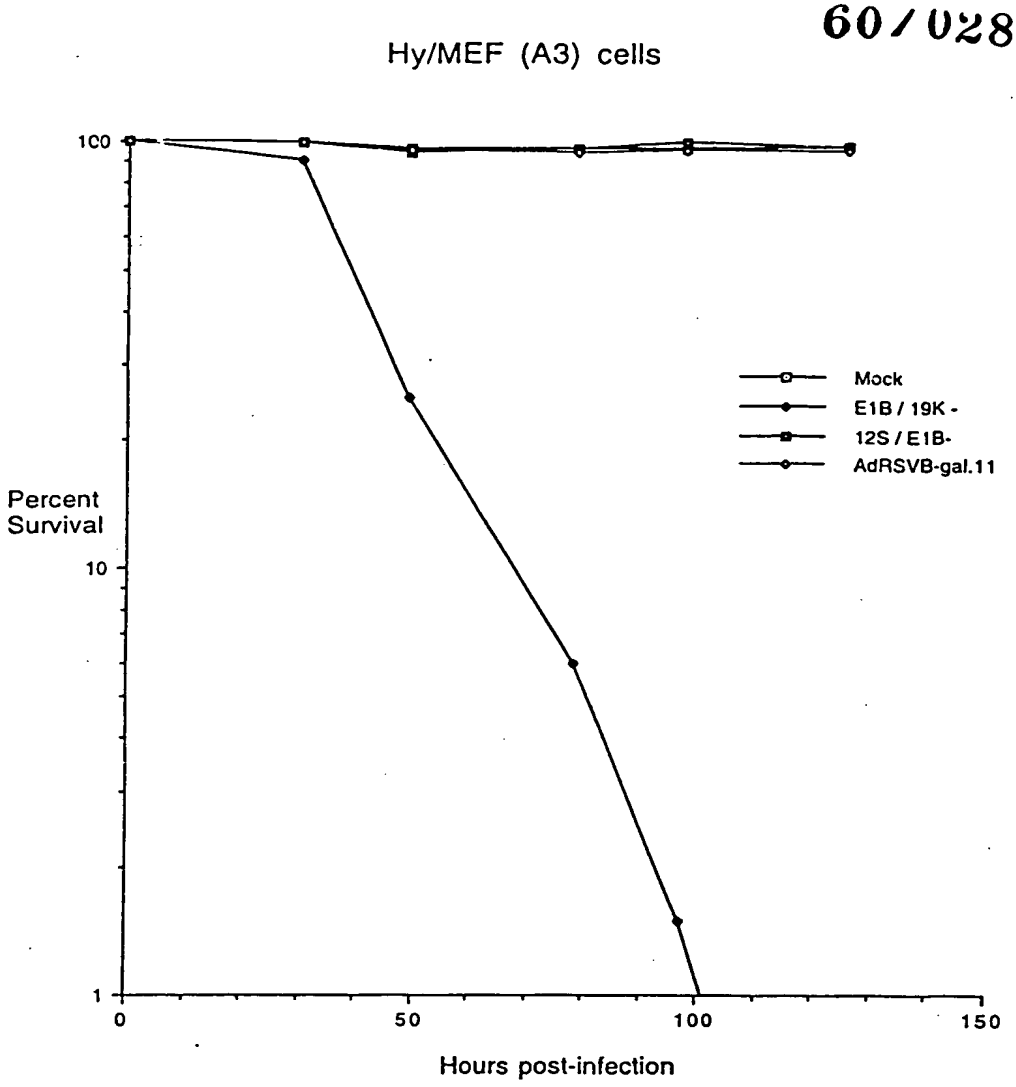

Hy/MEF (A3) cells
100
10
1
Percent Survival
0
50
100
150
Hours post-infection
Mock
E1B / 19K -
12S / E1B-
AdRSVB-gal.11

Fig. 9

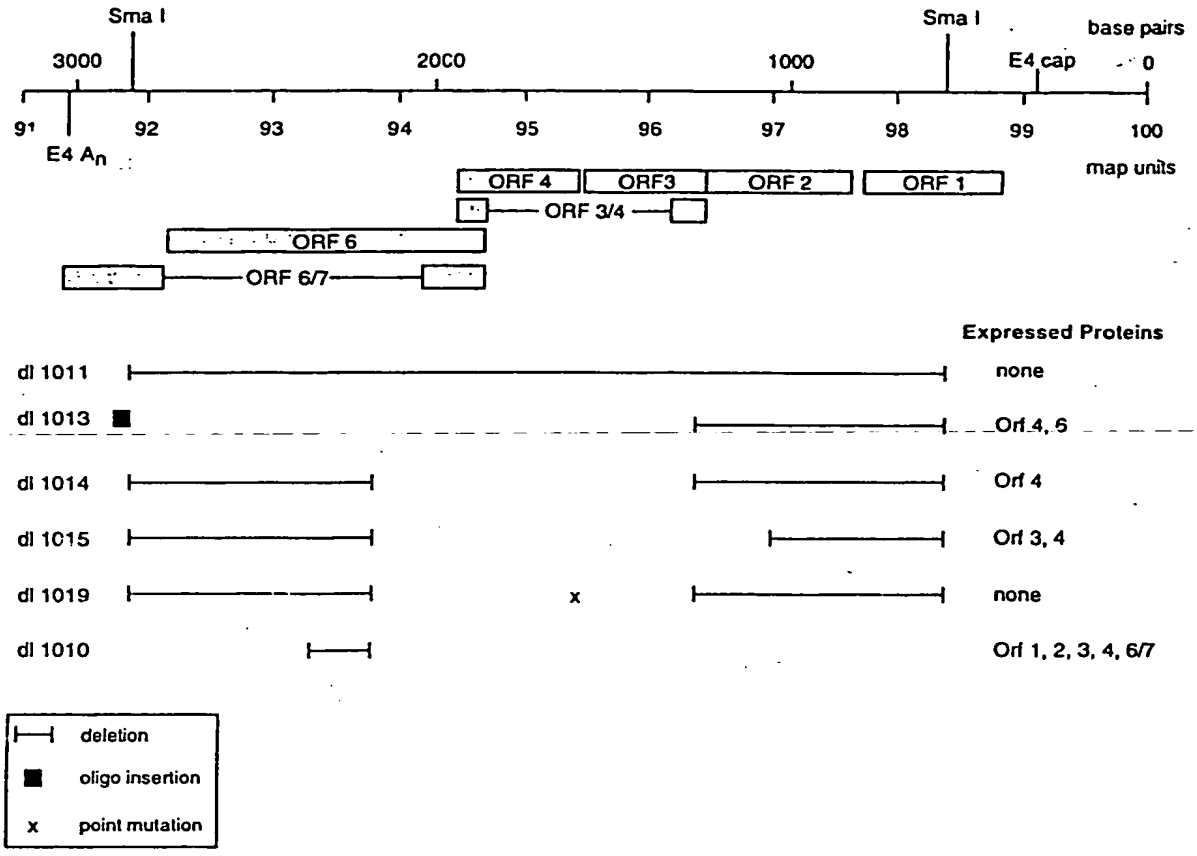

Sma I
Sma I
base pairs
3000
2000
1000
E4 cap
0
91
92
93
94
95
96
97
98
99
100
E4 An
map units
ORF 4
ORF3
ORF 2
ORF 1
ORF 3/4
ORF 6
ORF 6/7
Expressed Proteins
dl 1011
none
dl 1013
Orf 4, 6
dl 1014
Orf 4
dl 1015
Orf 3, 4
dl 1019
x
none
dl 1010
Orf 1, 2, 3, 4, 6/7
deletion
oligo insertion
x
point mutation

Fig. 10

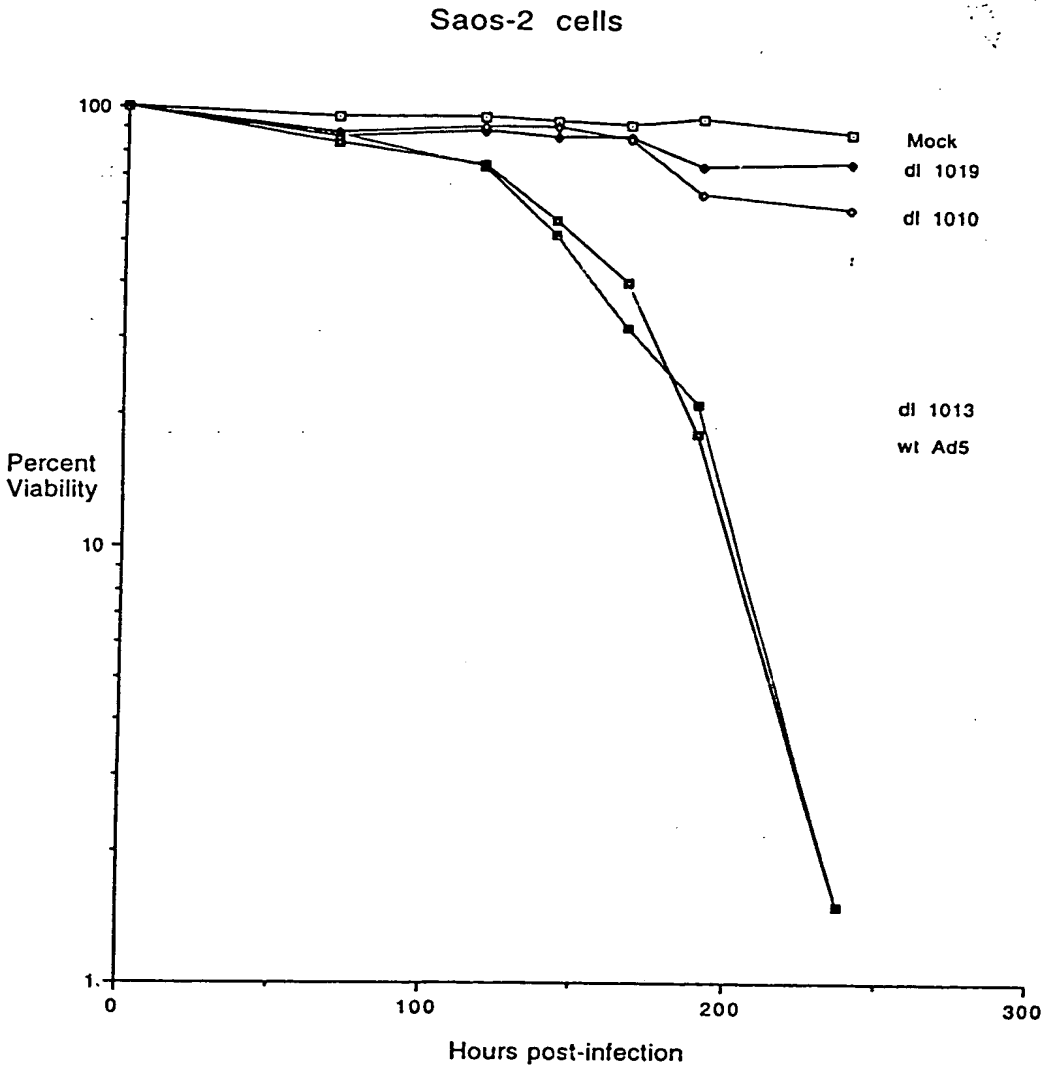

Saos-2 cells
100
10
1
Percent Viability
0
100
200
300
Hours post-infection
Mock
dl 1019
dl 1010
dl 1013
wt Ad5

Fig. 11

60/028740

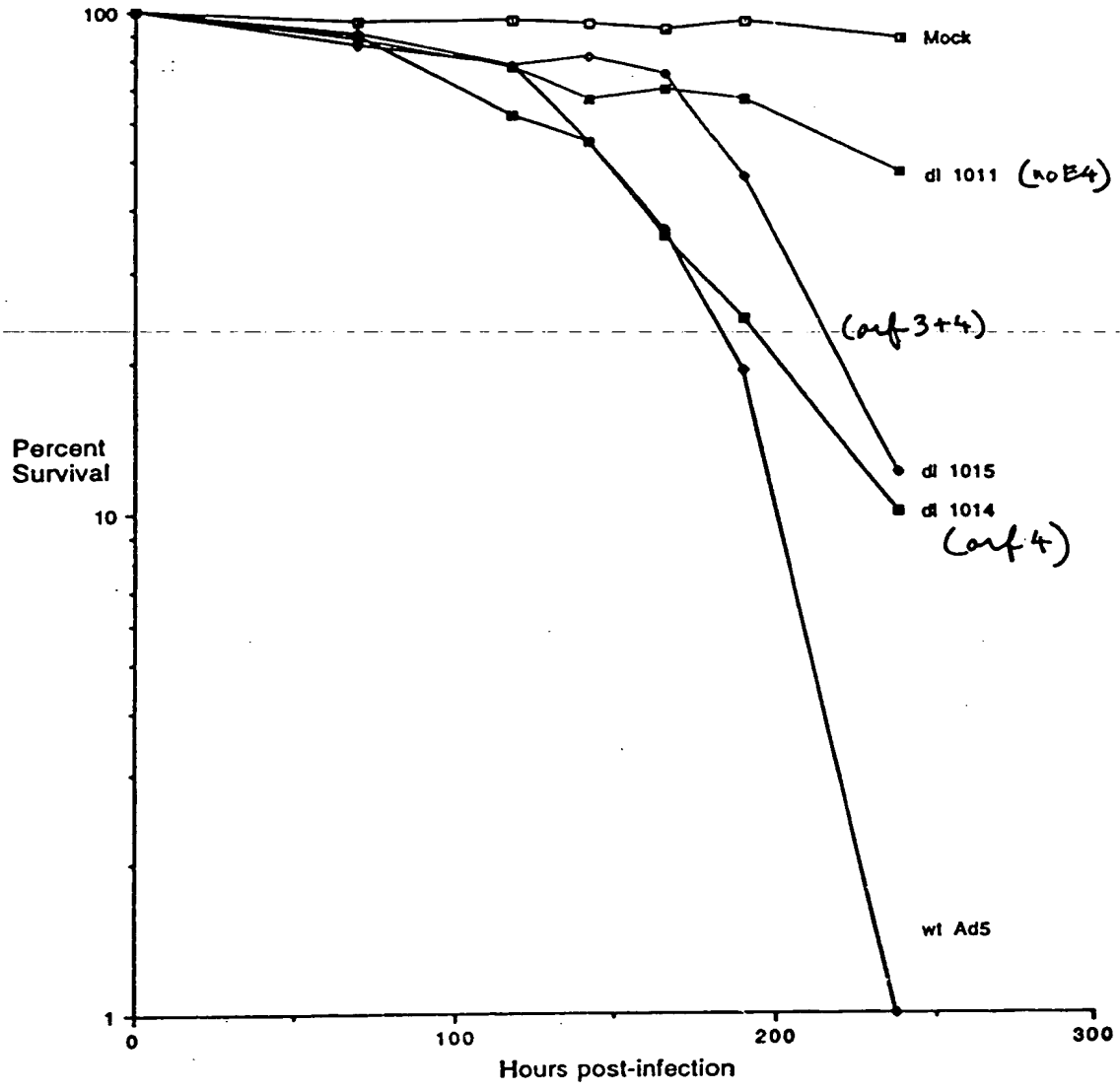
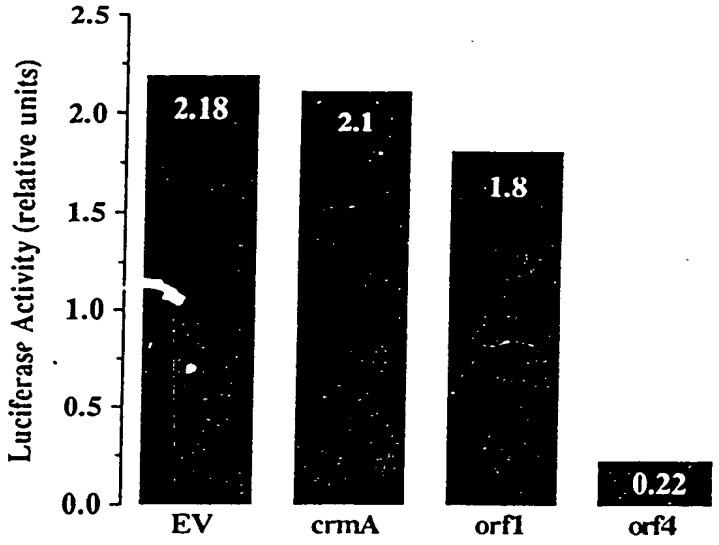


Fig. 12



Co-expression of pRSV-luciferase and pRSV-E4 proteins in p53-null mouse fibroblast A1A3 cells. Cells were transfected with control empty vector (EV, no protein produced) or vector encoding CrmA, E4orf4orf1, or E4orf4 together with a luciferase reporter gene. Cells were lysed 48 hours later and luciferase activity measured.

Fig. 13